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Original Contribution

ANTIOXIDANTS INHIBIT THE EXPRESSION OF INTERCELLULAR CELL ADHESION MOLECULE-1 AND VASCULAR CELL ADHESION MOLECULE-1 INDUCED BY OXIDIZED LDL ON HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

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Abstract—The oxidative modification of low density lipoprotein (LDL) and the endothelial expression of adhesion molecules are key events in the pathogenesis of atherosclerosis. In this study we evaluated the effect of oxidized LDL on the expression of intercellular cell adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin on human umbilical vein endothelial cells (HUVECs). The hypothesis that oxidized LDL functions as a prooxidant signal was also evaluated, by studying the effect of different radical-scavenging antioxidants on expression of adhesion molecules. LDL was oxidized by using Cu^{2+}, HUVECs or phospholipase A_2 (PLA_2)/soybean lipoxygenase (SLO), the degree of oxidation being measured as thiobarbituric acid-reactive substances (TBARS) and conjugated dienes (CD). Exposure of 200 μg/ml of native LDL to 1 μm Cu^{2+}, HUVECs and to PLA_2/SLO resulted in four- to fivefold higher levels of TBARS and CD than in native LDL. Cu^{2+}- (1 μM), HUVEC-, and PLA_2/SLO-oxidized LDL caused a dose-dependent, significant increase of ICAM-1 and VCAM-1 ($p < .01$). The expression of E-selectin did not change. LDL oxidized with a 2.5 and 5 μM Cu^{2+} did not increase ICAM-1 and VCAM-1 significantly. Both the Cu^{2+}- and HUVEC-oxidized LDL, subjected to dialysis and ultrafiltration, induced ICAM-1 and VCAM-1 expression. After incubation with the ultrafiltrate, the expression of ICAM-1 and VCAM-1 was not significantly different from that obtained with native LDL. LDL pretreated with different antioxidants (vitamin E and probucol) and subjected to oxidation by Cu^{2+} and HUVECs induced a significantly lower expression of ICAM-1 and VCAM-1 than nonloaded LDL ($p < .01$). The pretreatment of HUVECs with vitamin E and probucol significantly reduced the expression of VCAM-1 on HUVECs induced by oxidized LDL ($p < .01$); the effect on ICAM-1 was much less evident. In conclusion, oxidized LDL can induce the expression of different adhesion molecules on HUVECs; this induction can be prevented by pretreating either the LDL or the cells with radical-scavenging antioxidant.

Keywords—Adhesion molecules, Antioxidants, Atherosclerosis, Free radical, Human umbilical vein endothelial cells (HUVECs), Intercellular cell adhesion molecule-1 (ICAM-1), Oxidized LDL, Vascular cell adhesion molecule-1 (VCAM-1)

INTRODUCTION

An important early event in the initiation of atherosclerosis is the increased interaction of monocytes with endothelial cells lining the vessel wall.[1] The adhesion of leukocytes to the endothelium is mediated by cell adhesion molecules. These adhesion molecules, which include E-selectin (a specific product of endothelial cells), intercellular cell adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) (the product of both endothelial and other cells), may be important in controlling the extravasation of leukocytes out of the circulation in acute and chronic inflammation.[2,3] Endothelial cell activation by cytokines

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pression of these binding molecules. It has been shown that the signal-transduction pathways for these binding molecules induced by tumor necrosis factor-α (TNF) and interleukin-1β include the translocation of the transcription factor NF-κB.[4] Recent findings indicate the involvement of intracellularly generated oxygen-derived free radicals (ODFR) in this pathway of NF-κB activation.[5] Modulation of the expression of these adhesion molecules by ODFR may, therefore, be an important mechanism in initiating atherosclerosis.

Low-density lipoprotein (LDL) oxidation is believed to play an important role in the development of atherosclerotic plaque.[6-10] This view is supported by the presence in atheromatous lesions of LDL with characteristics similar to those of oxidatively modified LDL.[11] To date, the mechanism by which oxidized LDL can accelerate the atherogenic process is unknown.

Studies in culture cells have demonstrated that oxidized LDL is chemotactic for monocytes and stimulates monocyte endothelial interactions.[12,13] Parhami et al.,[14] however, found that only minimally modified LDL activated endothelial cells to increase their interaction with monocytes, while highly Cu^{2+}-oxidized LDL did not. The same authors have also ruled out any contribution of E-selectin, VCAM-1, and ICAM-1 to the increased monocyte binding after incubation of endothelial cells with modified LDL. Frostegard et al.[15] showed that Cu^{2+}-oxidized LDL was as effective as interleukin-1β in stimulating the ability of cultured human endothelial cells to bind U937 monocytic cells. Subsequently, the same authors[16] showed that oxidized LDL per se did not influence the expression of adhesion molecules and that only incubation of endothelial cells with conditioned medium from mononuclear cells grown in the presence of oxidized LDL resulted in an increase of ICAM-1 and VCAM-1. At variance with these results, Jeng et al.[17] found that Cu^{2+}-oxidized LDL enhanced monocyte-endothelial cell binding through cell surface expression of ICAM-1, but not VCAM-1. Recently, Khan et al.[18] observed that incubation of highly Cu^{2+}-oxidized LDL with endothelial cells did not induce VCAM-1 ICAM-1 or E-selectin expression, but augmented their cytokine-activated expression.

Because the discrepancy in these results may depend on differing methods for preparation of oxidized LDL, the present study analyzes the effect of LDL, oxidized in different ways (Cu^{2+}, endothelial cells, enzymatic), on the expression of ICAM-1, VCAM-1, and E-selectin in human umbilical vein endothelial cells (HUVECs). The study also addresses the problem whether the expression of adhesion molecules is a direct effect of onstrated effect of ODFR on modulation of adhesion molecule expression in endothelial cells,[5] this study also aimed to ascertain whether oxidized LDL functions as a prooxidant signal that can be counteracted by radical scavenging antioxidants.

MATERIALS AND METHODS

LDL isolation

Whole blood, obtained by venipuncture from healthy volunteeers after 12 h fasting, was collected into Vacutainer tubes containing ethylenediamine tetraacetic acid (EDTA) (1 mg/ml), immediately centrifuged at 2000 rpm for 20 min at 4°C, and processed for LDL separation within 1 d. LDL was isolated by sequential flotation in NaBr solution[19] containing 1 mg/ml EDTA (d 1.019–1.063 g/ml). To minimize oxidation of LDL during the isolation process, all solutions used in the isolation were deoxygenated by bubbling with argon. LDL was stored sterile in the dark, under nitrogen at 4°C, and used within 3 d.

Cell culture

HUVECs were isolated from human umbilical veins according to the method of Jaffe[20] and used at passages 2–4. The cells were grown in 75 cm^2 culture flasks (Falcon, Becton Dickinson, Lincoln Park, USA), filled with 10 ml of Ham's F-12 medium (Sigma, St. Louis, MO) containing 10% fetal calf serum (FCS) (Seromed, Berlin, Germany), 2 mM glutamine (Seromed, Berlin, Germany), 30 μg/ml endothelial cell growth supplement (Sigma), 100 μg/ml heparin (Sigma), 100 U/ml penicillin-streptomycin (Sigma), 100 μg/ml streptomycin (Sigma), and 2.5 μg/ml anphotericin (Sigma). The flasks were incubated at 37°C, 100% humidity and 5% of CO_2. The medium was refreshed every 2 d. At the beginning of each experiment the cells were detached by 0.01% trypsin/EDTA (Sigma). The trypsin was inactivated by dilution, and the cells were washed and counted. Cells were plated at a concentration of 20,000 cells/cm^2 on a multiwell plate (9.6 cm^2/well) (Falcon, Becton Dickinson, Lincoln Park, USA), grown for 3 d, and then used for the incubations. At that time the cells were nonconfluent ($0.26–0.40 \times 10^5$ cells/cm^2).

HUVECs were harvested and characterized in terms of to acetylated LDL binding and factor VIII expression, according to previously described techniques.[21]

LDL oxidation

concentrations of vitamin E (Sigma) and probucol (Sigma) were incorporated into LDL as previously described.[22] These substances were incubated at a final concentration of 5 μM with aliquots of the same LDL pool, for 3 h at room temperature. Each aliquot was then gel filtered on PD-10 disposable columns (Pharmacia, Upsala, Sweden) in 10 mM phosphate-buffered saline (PBS), pH 7.4, containing EDTA (1 mg/ml); this ensured elimination of any drug not incorporated into the lipoprotein. The drug incorporated into the LDL was then evaluated. LDL vitamin E and probucol were measured by HPLC with fluorescence detection (Gold system; Beckman Instruments Inc., Palo Alto, CA), as previously described.[23,24] Immediately before the oxidation incubations, LDL was separated from EDTA and from diffusible low molecular mass compounds by gel filtration on PD-10 disposable columns (Pharmacia) in 10 mM PBS, pH 7.4.

HUVEC-modified LDL was prepared by adding 1.5 ml of serum-free F-12 medium containing 200 μg/ml LDL protein to each well of HUVECs, and incubating for 24 h at 37°C. Cu^{2+}-modified LDL (200 μg protein/ml) was prepared by exposure of LDL to 1 to 5 μM $CuSO_4$ in serum-free F-12 medium for 24 h at 37°C. Enzymatically modified LDL was prepared with slight modifications from the method described by Sparrow et al.,[25] using soybean lipoxygenase (SLO) (Sigma), and phospholipase A_2 (PLA_2)(Sigma). SLO and PLA_2 were separately bound to CNBr-activated sepharose beads (Pharmacia) in coupling buffer (0.1 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). Approximately 1700 U of SLO and 4 U of PLA_2 were bound per mg of beads. LDL was separated from EDTA by gel filtration on PD-10 disposable columns (Pharmacia) in 10 mM PBS, pH 7.4. 0.5 mg of LDL in 10 mM PBS, pH 7.4, was incubated for another 24 h at 4°C, with 20 U of PLA_2, 5000 U of SLO, and 1 mM $CaCl_2$, in 12 × 75 mm sterile polypropylene snap cap tubes. Finally, the enzymes were removed by centrifugation, and 100 μM butylated hydroxytoluene and 0.3 mM EDTA were added.

Prior to addition to HUVECs, the supernatant of each LDL preparation was passed over a prepacked endotoxin affinity column (Detoxigel, Pierce, Rockford, IL).

To separate the effect of oxidized LDL from that of diffusible low mol mass compounds present in the medium after the oxidation, the incubation samples were also dialysed overnight against 3 l of PBS, pH 7.4, at 4°C; Spectra/por dialysis tubing closures were used (Spectrum, Houston, TX). The sample solution was then ultrafiltered through an anisotropic membrane (Centricon 30, Amicon, Beverly, MA).

The extent of LDL oxidation was determined by evaluating the level of thiobarbituric acid-reactive substances (TBARS). TBARS were expressed as malondialdehyde equivalents, as previously described.[26] Aliquots of the incubation mixture containing 100 μg of LDL were removed and added to tubes containing 0.05 ml of 2% butylated hydroxytoluene, 2 ml of 0.67 TBA, and 10% trichloroacetic acid (2:1) in 0.25 N HCl. After heating at 100°C for 10 min, the tubes were cooled and centrifuged at 2500 rpm for 10 min. The absorbance of the supernatant fraction was read at 532 nm, and the quantitation was achieved by comparison with a standard curve of malondialdehyde equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetraethoxypropane.

Lipid peroxidation products were also determined by HPLC analysis of saponified lipids, as previously described.[27] LDL and oxidized LDL lipids were extracted from the sample (0.25 mg protein) with chloroform-methanol-acetic acid 2:1:0.01. After removing the chloroform fraction, the dried lipids were reconstituted in 0.5 ml of ethanol; 10 N NaOH was then added at 60°C for 20 min. The solution was neutralized by the addition of 0.03 ml glacial acetic acid and dried under nitrogen. After mixing and centrifugation, 0.8 ml of the heptane layer was removed, dried under nitrogen, and resolubilized in 0.15 ml of heptane. Fatty acid oxidation products were separated, using a normal-phase silica column (Zorbax Sil, 25 cm × 4.6 mm, 5 μm) in a "Gold" system (Beckman Instruments Inc.); this included two pumps, an autosampler (Gilson, model 232 Bio, Biolabo Instruments, Milano, Italy), a PS2/50 IBM computer with the "Gold" acquisition, and processing data software. The mobile phase was a quaternary mixture of heptane-diethylether-isopropanol-acetic acid 100:10:0.9:0.1, at a flow rate of 2 ml/min. Conjugated dienes (CD) were monitored at 234 nm. Standards of linoleate hydroxides, 9- and 13 hydroxyoctadienoic acid (Cayman Chemical, Ann Arbor, MI) were used.

Protein was measured by the Pierce BCA protein assay reagent.[28]

Incubation of oxidized LDL with HUVECs

HUVEC monolayers were incubated with different concentrations of oxidized LDL, in M-199 medium containing 10% FCS and supplemented with 5 μg/ml polymixin B, for 24 h at 37°C. The time of incubation was chosen from preliminary time course studies indicating that ICAM-1 and VCAM-1 expression induced by 200 μg of oxidized LDL (1 μM Cu^{2+}) increased after 6 h, reached a plateau after 12 h and remained high for 36 h. At these times no significant expression of E-selectin was observed.

Some HUVEC monolayers were also pretreated for

18 h with equal concentrations (5 μM) of vitamin E and probucol, before the addition of oxidized LDL. Control incubations were also performed with different concentrations of native LDL.

Cytotoxicity evaluation

To assess cell survival, hexosaminidase, a stable cytosolic enzyme released by cells when they undergo lysis, was measured according to the method of Landegren.[29] Briefly, the substrate for the enzyme hexosaminidase, *p-nitrophenol*-N-acetyl-β-D-glucoseaminide (Sigma), was dissolved at 7.5 mM in 0.1 M citrate buffer, pH 5. The solution was then mixed with an equal volume of 0.5% Triton X-100 (Sigma) in water and added in volumes of 60 μl to cells for 60 min at 37°C. The reaction was blocked by addition of 50 mM glycine (Sigma) buffer, pH 10.4. Absorbance was measured in a Bio-Rad microplate reader (Model 450, Bio-Rad Laboratories S.r.L., Milan, Italy) at 405 nm. Results, expressed as percentage toxicity, were as $(E\text{-}S) \times 100/M\text{-}S$, where E represents the average absorbance readings from the experimental wells, S the average spontaneous release, and M the maximum release after cell lysis.

Cell adhesion molecule expression

Cell adhesion molecule expression was evaluated in a fluorescence-activated cell sorter. Cells were harvested by careful treatment with 0.01% trypsin/EDTA antagonized by immediate addition of 10% FCS. In these conditions, percentage cell toxicity was less than 3% as judged by hexosaminidase activity.[29] Cells (6×10^4) were treated for 30 min with saturating amounts of anti-ICAM-1, VCAM-1, and E-selectin monoclonal antibodies (Immunotech, Marseille, France) in PBS containing 0.5% bovine serum albumin (BSA). For staining, cells were incubated with goat antimouse fluorescein isothiocyanate IgG (Immunotech, Marseille, France). To avoid unspecific binding, cells were then preincubated with 5% human serum in PBS for 15 min on ice. Samples were washed twice with fluorescence-activated cell sorter buffer (Becton Dickinson, Heidelberg, Germany), fixed in 2% paraformaldehyde to prevent homotypic aggregation and analyzed with 10,000 cells per sample by a fluorescence-activated cell sorter (Becton Dickinson, Heidelberg, Germany). To correct for unspecific binding, values from nonspecific goat IgG (Immunotech, Marseille, France) were subtracted from each value obtained from staining with specific antibody.

Statistical analysis

Data on adhesion molecules are given as mean values ± SD and are expressed as % variation of the mean control fluorescence intensity (no addition of oxidized LDL). They represent the results of experiments performed in quadruplicate on 12 separate occasions, involving six different batches of LDL.

Statistical analysis was performed by analysis of variance and subsequent by post hoc analysis, using the "SYSTAT" program and statistical software manual (SYSTAT Inc., Evanston, IL) for Macintosh.

RESULTS

LDL oxidation

In our experimental conditions, exposure of 200 μg/ml of native LDL to 1 μM Cu^{2+}, HUVECs, and to PLA_2/SLO for 24 h resulted in four- to fivefold higher levels of TBARS and CD (Fig. 1) and greater mobility in agarose gel electrophoresis (data not shown) than observed with native LDL.

The exposure of HUVECs to medium containing 200 μg/ml of LDL oxidized with 2.5 and 5 μM Cu^{2+} resulted in a 9- to 14-fold increase of TBARS and in five- to sixfold increase in CD compared to native LDL. In passing from a concentration of 1 μM Cu^{2+} to 2.5 and 5 μM Cu^{2+}, the increase in TBARS (expressed as % variation) was far greater than that in CD ($p < .001$); TBARS increased from 3.21 ± 0.14 nmol/ml to 6.45 ± 0.32 nmol/ml (+100.0 ± 7.5%) and 10.98 nmol/ml (+242.1 ± 9.1%), and CD from 24.77 ± 2.46 nmol/ml to 31.99 ± 3.08 nmol/ml (+29.1 ± 4.2%) and to 38.89

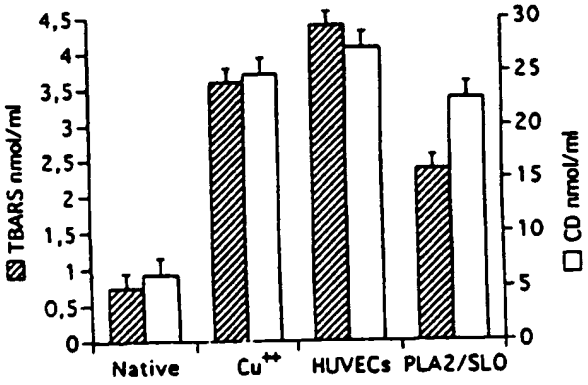

Fig. 1. Thiobarbituric acid-reactive substances (TBARS) and conjugated diene (CD) levels obtained after incubation of native LDL (0.2 mg LDL protein) with Cu^{2+} (1 μM), human umbilical vein endothelial cells (HUVECs) and phospholipase A_2 (PLA_2)/soybean lipoxigenase (SLO) for 24 h. Aliquots were taken for TBARS and CD determinations. TBARS and CD represent the results (mean ± SD) of experiments performed in quadruplicate on twelve separate occasions, involving six different batches of LDL.

± 4.16 nmol/ml (57.6 ± 6.1%) at 2.5 and 5 μM Cu^{2+}, respectively. Figure 2 shows the effect of the different Cu^{2+} concentrations, expressed in terms of Cu^{2+}/LDL protein ratio on TBARS and CD levels.

Effect of oxidized LDL on ICAM-1, VCAM-1, and E-selectin expression on HUVECs

To analyze the effect of oxidized LDL on ICAM-1, VCAM-1, and E-selectin, confluent cultures of HUVECs were exposed for 24 h to medium containing different amounts (from 50 to 200 μg/ml) of 1 μM Cu^{2+}-, HUVEC-, and PLA_2/SLO-oxidized LDL. Cu^{2+}-, HUVEC-, and PLA_2/SLO-oxidized LDL caused a dose-dependent, significant increase of ICAM-1 and VCAM-1. There was also a slight, nonsignificant increase in E-selectin. Figure 3 shows the effect of different concentrations of Cu^{2+}-, HUVEC-, and PLA_2/SLO-oxidized LDL on the expression of ICAM-1, VCAM-1, and E-selectin.

The exposure of HUVECs to medium containing 200 μg/ml of LDL oxidized with 2.5 and 5 μM Cu^{2+} did not significantly increase ICAM-1, VCAM-1, and E-selectin expression on HUVECs (Fig. 4).

The extent of endothelial cell cytotoxicity (expressed as % cytotoxicity), induced by 24 h incubation with LDL oxidized with 1 μM Cu^{2+}, HUVECs, and PLA_2/SLO, was always less than 3%. When incubated with LDL oxidized with 2.5 and 5 μM Cu^{2+} for 24 h, the percentage toxicity of endothelial cells was 16 ± 4% and 30 ± 5%, respectively.

Effect of dialysis and ultrafiltration of medium containing oxidized LDL on ICAM-1 and VCAM-1 expression on HUVECs

The potential contribution of substances in the oxidation medium to the induction of adhesion molecules by 1 μM Cu^{2+}- and HUVEC-oxidized LDL was examined by dialysis and ultrafiltrate experiments. An average of 58 and 66% of TBARS were removed from the LDL medium by separate experiments with dialysis and ultrafiltration, respectively. The Detoxigel column removed, on average, only 6% of TBARS. These various preparations of LDL, together with the ultrafiltrate, were tested for their ability to induce ICAM-1 and VCAM-1 on HUVECs. Figure 5 shows the effect of dialysis and ultrafiltration on the expression of ICAM-1 and VCAM-1 induced by oxidized LDL. Both the Cu^{2+}- and HUVEC-oxidized LDL, subjected to dialysis and ultrafiltration, induced ICAM-1 and VCAM-1 expression. After incubation with the ultrafiltrate, expression of ICAM-1 and VCAM-1 did not differ significantly from that obtained with native LDL.

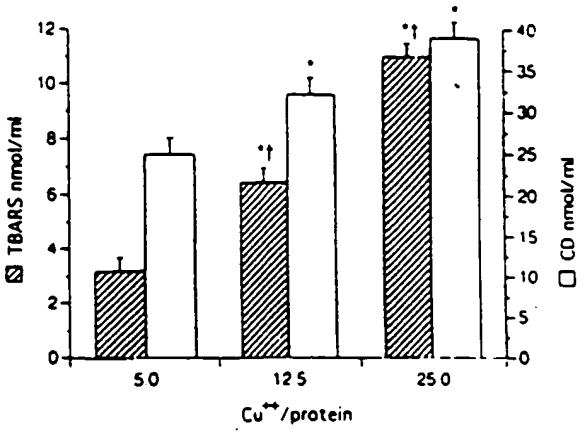

Fig. 2. Effect of Cu^{2+} to LDL protein ratio (Cu^{2+}/protein) on thiobarbituric acid-reactive substances (TBARS) and on conjugated diene (CD) levels. The same amount of LDL (0.2 mg LDL protein) was oxidized with 1, 2.5, and 5 μM Cu^{2+} for 24 h at 37°C. Aliquots were taken for TBARS and CD determination. The results represent the mean ± SD of experiments performed in quadruplicate on 12 separate occasions, involving six different batches of LDL. An asterisk indicates that the value differs from the Cu^{2+} to LDL protein ratio = 5 ($p < .01$). A dagger indicates that the effect for TBARS was significantly greater than for CD ($p < .001$).

Effect of antioxidants on LDL oxidation and ICAM-1 and VCAM-1 expression on HUVECs

We subsequently determined whether the presence of vitamin E and probucol in LDL during Cu^{2+}- and HUVEC-induced LDL oxidation was able to attenuate the expression of ICAM-1 and VCAM-1 on HUVECs. Table 1 shows the concentration of LDL vitamin E and probucol after incubation of LDL with equal concentrations (5 μM) of the two antioxidants for 18 h. The concentration of LDL vitamin E represents the net content of vitamin E incorporated into the lipoprotein (total LDL vitamin E − vitamin E concentration in untreated LDL). In these conditions, exposure of 200 μg/ml of LDL pretreated with antioxidants to Cu^{2+} and HUVECs resulted in 1.5–2.5-fold higher levels of TBARS and CD than obtained with native LDL (Table 1). The increase was significantly lower than that obtained with nonpretreated LDL ($p < .01$) under the same experimental conditions. Figure 6 shows the effect of Cu^{2+}- and HUVEC-oxidized LDL, pretreated with antioxidants, on ICAM-1 and VCAM-1 expression on HUVECs. The presence of antioxidants during lipoprotein oxidation significantly reduced the expression of oxidized LDL-induced ICAM-1 and VCAM-1, with no significant change in percentage cytotoxicity.

Effect of oxidized LDL on ICAM-1 and VCAM-1 expression on HUVECs pretreated with antioxidants

HUVEC monolayers were also pretreated with 5 μM vitamin E and probucol for 18 h before the addition of

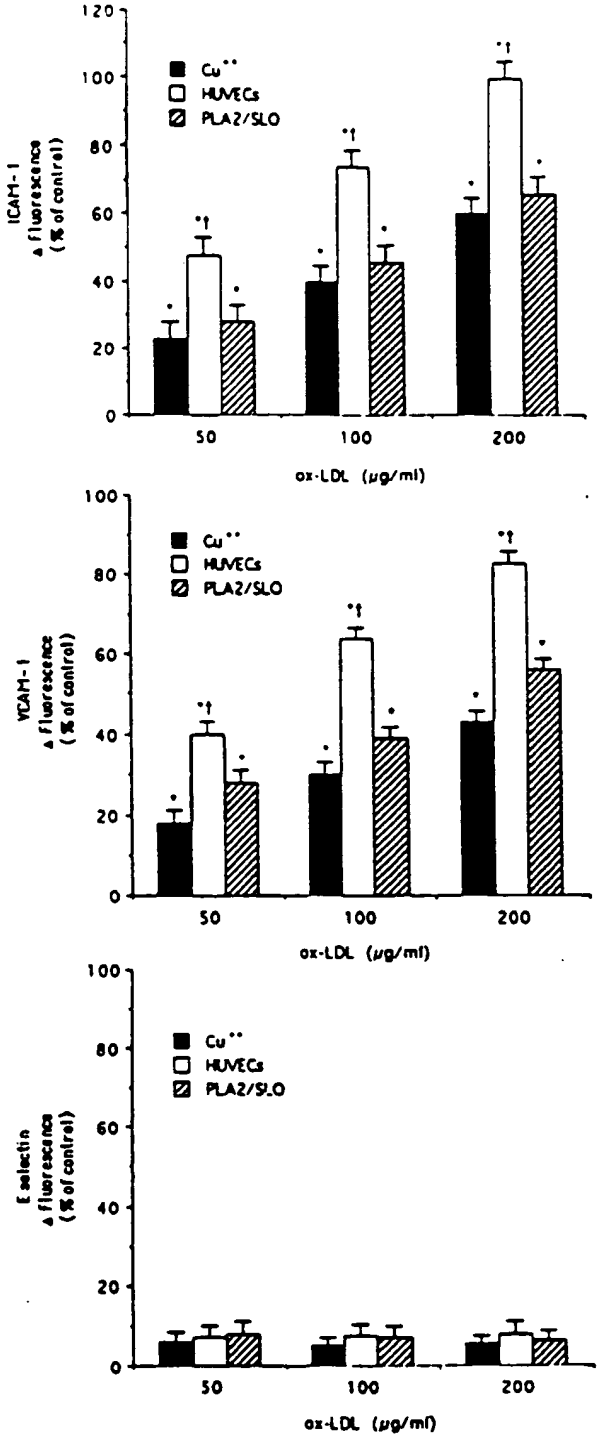

Fig. 3. Effect of different concentrations of Cu^{2+}-human umbilical vein endothelial cell (HUVEC)- and phospholipase A_2/soybean lipoxigenase (PLA_2/SLO)-oxidized LDL on the expression of ICAM-1, VCAM-1, and E-selectin on HUVECs. Cultures of HUVECs were exposed to medium containing different amounts (from 50 to 200 μg/ml) of 1 μM Cu^{2+}-HUVEC- and PLA_2/SLO-oxidized LDL. Cell adhesion molecule expression was evaluated in a fluorescence-activated cell sorter. Results are given as % variation of the control

oxidized LDL. Pretreatment of LDL or HUVECs with vitamin E and probucol did not induce any cytotoxicity. Control incubations were also performed with different concentrations of native LDL. Both antioxidants significantly reduced the expression of ICAM-1 and VCAM-1 induced by oxidized LDL (Fig. 7). This effect, however, was significantly greater for VCAM-1 than for ICAM-1 after incubation with both Cu^{2+}- and HUVEC-oxidized LDL ($p < .01$) (Fig. 7).

DISCUSSION

The results of this study show that oxidized LDL, irrespective of the method of oxidation, determined a dose-dependent increase in the expression of ICAM-1 and VCAM-1 on HUVECs. Cu^{2+}-, HUVEC-, and PLA_2/SLO oxidized LDL, on the contrary, did not significantly modify the expression of E-selectin on HUVECs. Our results are partially in agreement with those obtained under different experimental conditions by Jeng et al.,[17] who reported a 2.6-fold increase in cell surface expression of ICAM-1, but not VCAM-1, after treatment of HUVECs with Cu^{2+}-oxidized LDL. Consistent with the results of Parhami et al.,[14] this study also shows that the PLA_2/SLO-oxidized LDL did not increase the surface expression of E-selectin. Our results are at variance with those of Frostegard et al.,[16] who demonstrated that human mononuclear leukocytes exposed to low concentrations of Cu^{2+}-oxidized LDL secreted one or several factors stimulating the endothelial expression of cellular adhesion molecules and the ability of endothelial cells to bind monocytic cells; no such effect was reported for oxidized LDL per se. The results of this study also disagree with those of Khan et al.[18] who observed that incubation of highly Cu^{2+}-oxidized LDL with endothelial cells did not induce VCAM-1 ICAM-1 or E-selectin expression but augmented their cytokine-activated expression.

The difference between the above results and ours may be related to differences in the oxidized LDL preparations, for which the composition of lipid oxidation products can differ greatly. While there are no published data with which to compare the results on ICAM-1 and VCAM-1 expression induced by HUVEC-and PLA_2/SLO-oxidized LDL, for Cu^{2+}-oxidized LDL their expression seems to be determined by the amount of Cu^{2+} and, above all, the Cu^{2+} to LDL protein ratio. The same amount of LDL protein, incu-

Fig. 3 *Continued.* (no addition of oxidized LDL) and represent the mean ± SD of experiments performed in quadruplicate on 12 separate occasions, involving six different batches of LDL. An asterisk indicates that the value differs from the control ($p < .01$). A small cross indicates that the value obtained with HUVECs differs from that obtained with Cu^{2+} and PLA_2/SLO ($p < .01$).

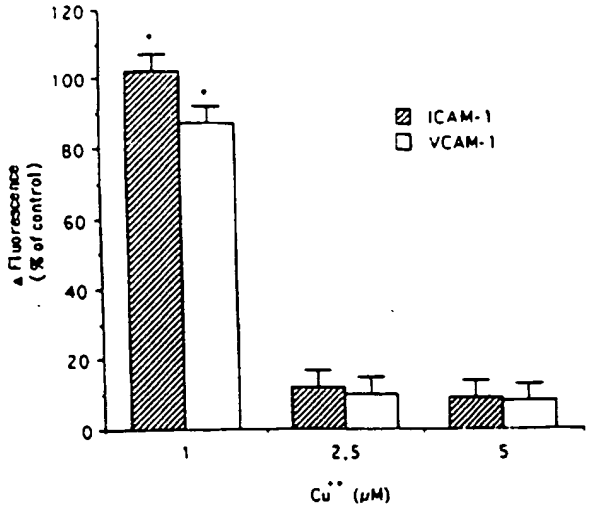

Fig. 4. Effect of LDL (0.2 mg protein) oxidized with 1, 2.5, and 5 μM Cu^{2+} on the expression of ICAM-1 and VCAM-1 on human umbilical vein endothelial cells (HUVECs). Cultures of HUVECs were exposed to medium containing LDL oxidized with different concentrations of Cu^{2+}. Cell adhesion molecule expression was evaluated in a fluorescence-activated cell sorter. Results are given as % variation of the control (no addition of oxidized LDL) and represent the mean ± SD of experiments performed in quadruplicate on 12 separate occasions, involving six different batches of LDL. An asterisk indicates that the value differs from 2.5 and 5 Cu^{2+} ($p < .01$).

bated with increasing amounts of Cu^{2+}, did not induce the expression of ICAM-1 and VCAM-1 on HUVECs. There was an associated increase in TBARS levels and in CD even though 2.5 and 5 μM Cu^{2+} induced disproportionally higher TBARS contents. These results can be explained by the ability of Cu^{2+} to effectively cleave lipid hydroperoxides to aldehydic products.[30] The decomposition of lipid hydroperoxides to aldehydes is a general phenomenon during lipid peroxidation in biological systems, and the amount of aldehydes that can be measured in oxidized LDL depends on the experimental conditions.[31] LDL oxidized for 24 h in the absence of Cu^{2+} in a dialysis bag was previously demonstrated to contain 17.8 mol aldehyde/mol LDL,[31] while LDL oxidized for 3 h in the presence of 1.6 μM Cu^{2+} contained 100 mol aldehyde/mol LDL.[32] It is, therefore, possible that, when the Cu^{2+} to LDL protein ratio is disproportionately high, there is a prevalence of oxidation products derived from the lipid hydroperoxides. On the other hand, when the ratio is lower, only a minor amount of CD can be cleaved to other oxidative products.

It is interesting to point out that the authors[16,18] who did not find any variation in the expression of adhesion molecule by Cu^{2+}-oxidized LDL used a Cu^{2+} to LDL protein ratio 10 times higher (ratio 50) than ours (ratio 5). On the basis of the present data, it is, of course, difficult to draw any definite conclusion as to why LDL oxidized with higher levels of Cu^{2+} did not induce the expression of ICAM-1 and VCAM-1 on HUVECs. Because the most markedly modified preparations of LDL increased cytotoxicity with the overall profile comparing favorably with TBARS levels, one explanation of our results could be that higher levels of Cu^{2+} might have been toxic to the HUVECs over 24 h of incubation.

The potential contribution of oxidized LDL components or substances in the oxidation medium to eliciting adhesion molecule expression on HUVECs was

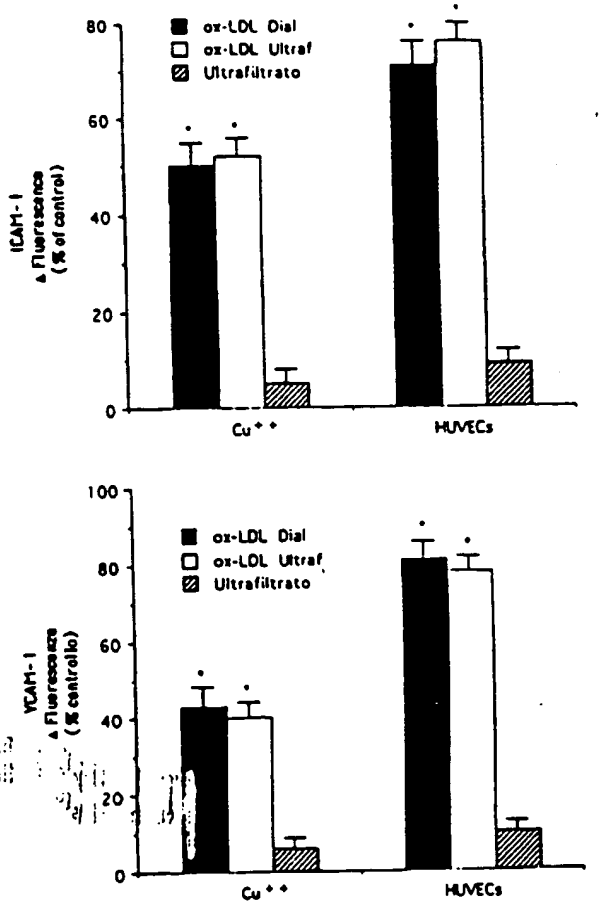

Fig. 5. Effect of dialysis (Dial) and ultrafiltration (Ultraf) of Cu^{2+}- and human umbilical vein endothelial cell (HUVEC)-oxidized (ox) LDL on the expression of ICAM-1 and VCAM-1 on HUVECs. LDL was dialyzed and ultrafiltered as described in Materials and Methods. Cultures of HUVECs were exposed to medium containing 200 μg/ml of 1 μM Cu^{2+}- and HUVEC-oxidized LDL. Cell adhesion molecule expression was evaluated in a fluorescence-activated cell sorter. Results are given as % variation of the control (no addition of oxidized LDL) and represent the mean ± SD of experiments performed in quadruplicate on 12 separate occasions, involving six different batches of LDL. An asterisk indicates that the value differs from the control ($p < .01$).

Table 1. Effect of Vitamin E and Probucol on Thiobarbituric Acid Reactive Substances (TBARS) and Conjugated Diene (CD) Production During Cu^{2+}- and HUVEC-Induced LDL Oxidation

		(nmol/ml)					
		Native		Cu^{2+}		HUVECs	
Added	Found (ng/mg LDL)	TBARS	CD	TBARS	CD	TBARS	CD
Vitamin E (5 μM)	428 ± 18	0.71 ± 0.09	1.85 ± 0.15	1.22 ± 0.11*	3.56 ± 0.24*	1.28 ± 0.13*	3.84 ± 0.31*
Probucol (5 μM)	323 ± 19	0.78 ± 0.07	1.72 ± 0.16	1.71 ± 0.15*	3.99 ± 0.29*	1.84 ± 0.14*	4.11 ± 0.37*
No addition	—	0.75 ± 0.10	1.79 ± 0.16	3.65 ± 0.14	7.14 ± 0.71	3.88 ± 0.16	7.84 ± 0.72

* Significantly lower than untreated LDL (no addition) $p < .01$.

To incorporate the antioxidants into the lipoprotein, equal concentrations of vitamin E and probucol were incubated with LDL for 3 h at room temperature. After gel-filtration the drug incorporated into the LDL was measured (see Materials and Methods). Vitamin E found represents the net amount of vitamin E incorporated (total vitamin E found − vitamin E present in untreated LDL). Treated and untreated LDLs were oxidized by Cu^{2+} and HUVECs as described in Materials and Methods.

studied with dialysis and ultrafiltration experiments. Only the Cu^{2+}- and HUVEC-oxidized LDL subjected to dialysis and ultrafiltration induced expression of ICAM-1 and VCAM-1; the ultrafiltrate did not. This observation is consistent with the finding that most of the products measured as TBARS, presumably cleaved products of hydroperoxides, were lost upon dialysis and ultrafiltration without any significant effect on adhesion molecule expression. It also indicates that, when LDL is modified slightly with low levels of Cu^{2+} (1 μM) and not ultrafiltered or dialyzed, the cleaved products of hydroperoxides have no appreciable effect on adhesion molecule expression. These results do not allow us to identify the LDL components that can stimulate adhesion molecule expression on HUVECs, and this point requires further study.

The results of the present study also show that LDL pretreated with different antioxidants and subjected to oxidation by Cu^{2+} and HUVECs induced a significantly lower expression of ICAM-1 and VCAM-1 on HUVECs than nonpretreated LDL. The results are in agreement with previous data showing that antioxidants increase the resistance of LDL to oxidation.[32,33] In vitro susceptibility of LDL to oxidation is associated with coronary atherosclerosis in human beings,[34,35] and LDL oxidation may also contribute to the atherosclerosis associated with hypertension.[36] Prevention or minimization of LDL oxidation by antioxidant therapy could protect against the expression of adhesion molecules induced by oxidized LDL, as well as other biologic effects.

Finally, the results of this study also demonstrated that the pretreatment of HUVECs with vitamin E and probucol significantly reduced the expression of VCAM-1 on HUVECs induced by oxidized LDL; the effect on ICAM-1 was much less evident. The nature of the mechanism by which vitamin E and probucol reduce the expression of VCAM-1 induced by oxidized LDL remains to be established. It has recently been demonstrated that some radical-scavenging antioxidants inhibit monocyte adhesion by suppressing nuclear factor (NF)-κB mobilization and induction of VCAM-1 in endothelial cells stimulated by TNF.[37,38] Because NF-κB activation by TNF was found to be controlled by reactive oxygen intermediates (ROIs),[5] it was suggested that radical-scavenging antioxidants preferentially exert their effect by scavenging radicals and altering intracellular thiol levels.[5,37-38] In our study, oxidized LDL may have influenced gene expression by causing oxidative stress and NF-κB activation; the radical-scavenging antioxidants used in this study may have prevented NF-κB–mediated adhesion molecule induction by exerting their effects on ROIs. This hypothesis is consistent with the recent finding that the ability of endothelial cells to express VCAM-1 in response to a cytokine signal may be modulated by oxidized LDL through modification of its redox-sensitive mode of regulation.[18]

Both vitamin E and probucol exerted a preferential effect on VCAM-1 expression induced by Cu^{2+}- and HUVEC-oxidized LDL. On the basis of the present results, it is, of course, difficult to explain why vitamin E and probucol affected ICAM-1 expression induced by Cu^{2+}- and HUVEC- oxidized LDL to a far lower extent than VCAM-1. It is worth noting that the results we obtained with vitamin E and probucol are similar to those obtained with a variety of cytokine inducers.[37,38] In response to these compounds, endothelial cells can concurrently activate the expression of the adhesion molecule genes VCAM-1, ICAM-1, and E-selectin.[37] It is now well established that only the activation of endothelial cell VCAM-1 gene expression is clearly regulated by a signal tranduction mechanism sensitive to inhibition by antioxidants.[37,38] Although a detailed description of E-selectin and ICAM-1 gene activation

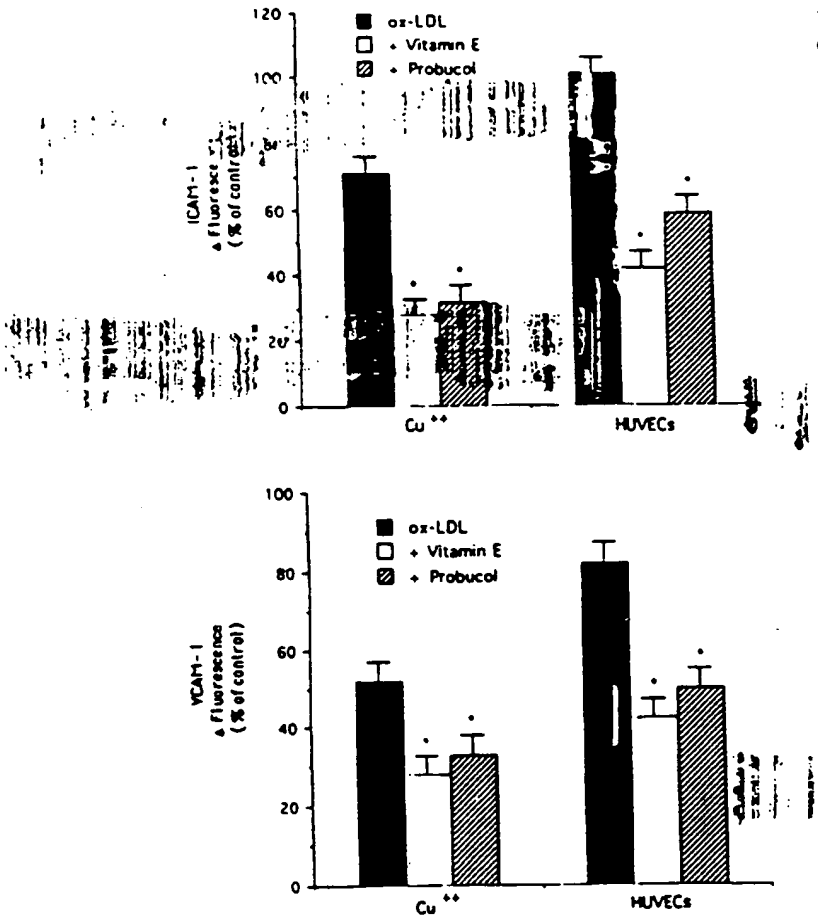

Fig. 6. Effect of Cu^{2+}- and human umbilical vein endothelial cell (HUVEC)-oxidized LDL, preloaded with vitamin E and probucol, on ICAM-1 and VCAM-1 expression on HUVECs. Vitamin E and probucol were incubated at a concentration of 5 μM with aliquots of the same LDL pool for 3 h at room temperature. Cultures of HUVECs were exposed to medium containing 200 μg/ml of 1 μM Cu^{2+}- and HUVEC-pretreated oxidized LDL. Cell adhesion molecule expression was evaluated in a fluorescence-activated cell sorter. Results are given as % variation of the control (no addition of oxidized LDL) and represent the mean ± SD of experiments performed in quadruplicate on 12 separate occasions, involving six different batches of LDL. An asterisk indicates that the value differs from the control ($p < .01$).

in endothelial cells is beyond the scope of this study, it is reasonabaly established that E-selectin and ICAM-I gene expression induced by cytokine inducers can escape inhibition by antioxidants.[37,38] It has, therefore, been hypothesised that E-selectin, VCAM-1, and ICAM-I may share common regulatory signals immediately after receptor activation by cytokine inducers, but that E-selectin and ICAM-I gene expression may be modulated by gene-specific signal transduction mechanisms.[37] A tentative explanation of our results could, therefore, be that oxidized LDL activate VCAM-I and ICAM-I gene expression and that, like with cytokine inducers, ICAM-gene expression escapes inhibition by antioxidants.

In our study, oxidized LDL did not induce the expression of E-selectin on HUVECs. This may imply that E-selectin, VCAM-1, and ICAM-1 do not share common regulatory signals immediately after receptor activation when stimulated by oxidized LDL.

In conclusion, the results of this study show that

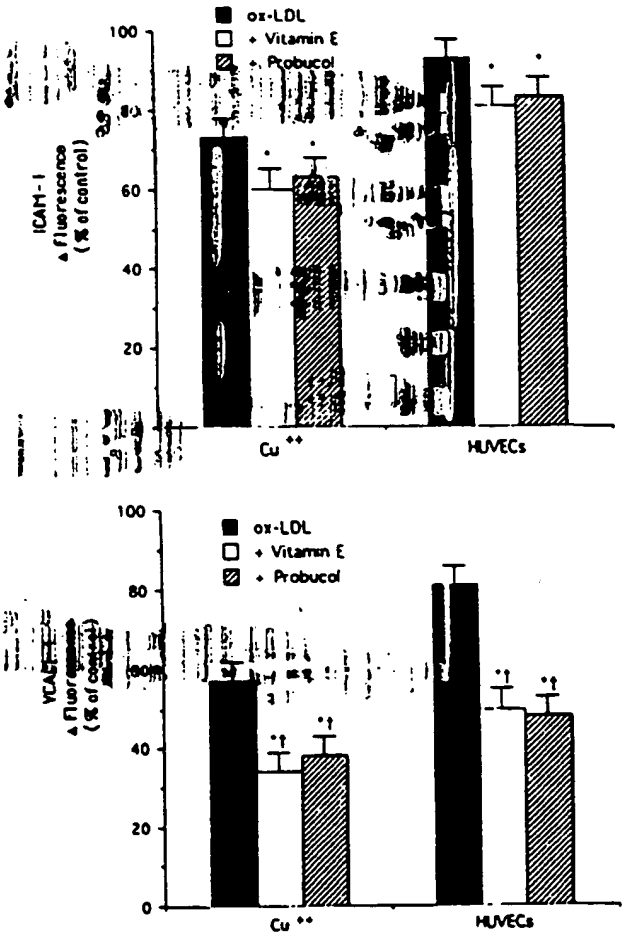

Fig. 7. Effect of pretreatment of human umbilical vein endothelial cells (HUVECs) with vitamin E and probucol on the oxidized LDL-induced expression of ICAM-1 and VCAM-1. HUVEC monolayers were pretreated with 5 μM vitamin E and probucol for 18 h before the addition of oxidized LDL. Cultures of pretreated HUVECs were exposed to medium containing 200 μg/ml of 1 μM Cu^{2+}-, HUVEC-oxidized LDL. Cell adhesion molecule expression was evaluated in a fluorescence-activated cell sorter. Results are given as % variation of the control (no addition of oxidized LDL) and represent the mean ± SD of experiments performed in quadruplicate on 12 separate occasions, involving six different batches of LDL. An asterisk indicates that the value differs from the control ($p < .01$). A small cross indicates that the effect for VCAM-1 was significantly greater than for ICAM-1 ($p < .01$).

oxidized LDL can induce the expression of different adhesion molecules on HUVECs, and that this induction can be prevented by pretreating either the LDL or the cells by radical-scavenging antioxidants. Because monocyte recruitment into the vascular wall following adhesion to endothelial cells is a crucial step in the pathogenesis of atherosclerosis, our study implies that antioxidants may have as yet unexplored therapeutic effects in treatment of this condition.

REFERENCES

1. Ross, R. The pathogenesis of atherosclerosis—An update. *N. Engl. J. Med.* 314:488–500; 1986.
2. Bevilacqua, M. P. Endothelial-leukocyte adhesion molecules. *Annu. Rev. Immunol.* 11:767–804; 1993.
3. Springer, T. A. Adhesion receptors of the immune system. *Nature* 346:425–434; 1990.
4. Lenardo, M. J.; Baltimore, D. NF-κB: A pleiotropic mediator of inducible and tissue-specific gene control. *Cell* 58:227–229; 1989.
5. Satriano, J. A.; Shuldiner, M.; Hora, K.; Xing, Y.; Schlondorff, D. Oxygen radicals as second messengers for expression of the monocyte chemoattractant protein, JE/MCP-1, and the monocyte colony-stimulating factor, CSF-1, in response to tumor necrosis factor-alpha and immunoglobulin G. *J. Clin. Invest.* 92:1564–1571; 1993.
6. Avogaro, P.; Bittolo Bon, G.; Cazzolato, G. Presence of a modified low density lipoprotein in humans. *Arteriosclerosis* 8:79–87; 1988.
7. Boyd, H. C.; Gown, A. M.; Wolfbauer, G.; Chait, A. Direct evidence for a protein recognized by a monoclonal antibody against oxidatively modified LDL in atherosclerotic lesions from a Watanabe heritable hyperlipidemic rabbit. *Am. J. Pathol.* 135:815–824; 1989.
8. Steinberg, D.; Parthasarathy, M. D.; Carew, T. E.; Khoo, J. C.; Witztum, J. L. Beyond cholesterol. Modification of low-density lipoprotein that increases its atherogenicity. *N. Engl. J. Med.* 320:915–924; 1989.
9. Rosenfeld, M. E. Oxidized LDL affects multiple atherogenic cellular responses. *Circulation* 83:2137–2140; 1991.
10. Wiklund, O.; Mattson, L.; Bjornheden, T.; Camejo, G.; Bondjers, G. Uptake and degradation of low density lipoproteins in atherosclerotic rabbit aorta: Role of local LDL modification. *J. Lipid Res.* 32:55–62; 1991.
11. Palinsky, W.; Yla-Herttuala, S.; Rosenfeld, M. E.; Butler, S. W.; Socher, S. A.; Parthasarathy, S. Antisera and monoclonal antibodies specific for epitopes generated during oxidative modification of low density lipoprotein. *Arteriosclerosis* 10:325–332; 1990.
12. Quinn, M.; Parthasarathy, S; Fong, L. G.; Steinberg, D. Oxidatively modified low density lipoproteins: A potential role in recruitment and retention of monocyte/macrophages during atherogenesis. *Proc. Natl. Acad. Sci. USA* 84:2995–2998; 1987.
13. Berliner, J. A.; Territo, M. C.; Sevanian, A.; Ramin, S.; Kim, J. A.; Bamshad, B.; Esterson, M.; Fogelman, A. M. Minimally modified low-density lipoprotein stimulates monocyte endothelial interactions. *J. Clin. Invest.* 85::1260–1266; 1990.
14. Parhami, F. F.; Fang, Z. T.; Fogelman, A. M.; Andalibi, A.; Territo, M. C.; Berliner, J. A. Minimally modified low density lipoprotein-induced inflammatory responses in endothelial cells are mediated by cyclic adenosine monophosphate. *J. Clin. Invest.* 92:471–478; 1993.
15. Frostegard, J.; Haegerstrand, A.; Gidlund, M.; Nilsson, J. Biologically modified LDL increases the adhesive properties of endothelial cells. *Atherosclerosis* 90:119–126; 1991.
16. Frostegard, J., Wu, R.; Haegerstrand, A.; Patarroyo, M.; Lefvert, A. K.; Nilsson, J. Mononuclear leukocytes exposed to oxidized low density lipoprotein secrete a factor that stimulates endothelial cells to express adhesion molecules. *Atherosclerosis* 103:213–219; 1993.
17. Jeng, J. R.; Chang, C. H.; Shieh, S. M.; Chiu, H. C. Oxidized low-density lipoprotein enhances monocyte-endothelial cell binding against shear-stress-induced detachment. *Biochim. Biophys. Acta* 1178:221–227; 1993.
18. Khan, B. V.; Parthasarathy, S. S.; Alexander, R. W.; Medford, R. M. Modified low density lipoprotein augment cytokine-activated vascular cell adhesion molecule-1 gene expression in human vascular endothelial cells. *J. Clin. Invest.* 95:1262–1270; 1995.
19. Havel, R. J.; Eder, M. A.; Bragdon, J. M. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* 34:1345–1353; 1955.
20. Jaffe, E. A.; Nachman, R. L.; Becker, C. G.; Minick, C. R Culture of human endothelial cells derived from umbilical veins. *J. Clin. Invest.* 52:2745–2752; 1973.
21. Smith, C. W.; Marlin, S. D.; Rothlein, R.; Toman, C.; Anderson, D. C. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophilis in vitro. *J. Clin. Invest.* 83:2008–2017; 1989.
22. Cominacini, L.; Garbin, U.; Cenci, B.; Davoli, A.; Pasini, C.; Ratti, E.; Gaviraghi, G.; Lo Cascio, V.; Pastorino, A. M. Predisposition to LDL oxidation during copper-catalyzed oxidative modification and its relation to alpha-tocopherol content in humans. *Clin. Chim. Acta* 204:57–68; 1991.
23. Lehmann, J.; Martin, H. L. Improved direct determination of alpha- and gamma-tocopherol in plasma and platelets by liquid chromatography, with fluorescence detection. *Clin. Chem.* 28:1345–1353; 1982.
24. Satonin, D. K.; Coutant, J. E. Comparison of gas chromatography and high performance liquid chromatography for the analysis of probucol in plasma. *J. Chromatogr.* 380:411–416; 1986.
25. Sparrow, C. P.; Parthasarathy, S.; Steinberg, D. Enzymatic modification of low density lipoprotein by purified lipoxygenase plus phospholipase A_2 mimics cell-mediated oxidative modification. *J. Lipid Res.* 29:745–755; 1988.
26. Aust, S. D. Lipid peroxidation. In: Greenwald, R. A. ed. *Handbook of methods for oxygen radical research.* Boca Raton, FL: CRC Press, Inc.; 1985:203–207.
27. Craig, E. T.; Jackson, R. L.; Ohlweiler, D. F.; Ku, G. Multiple lipid oxidation products in low density lipoproteins induce interleukin-1 beta release from human blood mononuclear cells. *J. Lipid Res.* 35:417–427; 1994.
28. Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, B. J.; Klenk, D. C. Measurement of protein using bicinchoninic acid. *Anal. Biochem.* 105:293–299; 1985.
29. Landegren, U. Measurement of cell numbers by means of the endogenous enzyme hexosaminidase: Application to detection of lymphokines and cell surface antigens. *J. Immunol. Methods* 67:379–385; 1984.
30. Esterbauer, H.; Rotheneder, M.; Striegl, G.; Waeg, G.; Ashy, A.; Sattler, W.; Jurgens, G. Vitamin E and other lipophilic antioxidants protect LDL against oxidation. *Fat Sci. Technol.* 91:316–324; 1989.
31. Esterbauer, H.; Gebicki, J.; Puhl, H.; Jurgens, G. The role of lipid peroxidation and antioxidants in oxidative modifications of LDL. *Free Radic. Biol. Med.* 13:341–390; 1992.
32. Esterbauer, H.; Quehenberger, O.; Jurgens, G. Oxidation of human low density lipoprotein with special attention to aldehydic lipid peroxidation products. In: Rice-Evans, C.; Halliwell, B. eds. *Free radicals: Methodology and concepts.* London: The Richelieu Press; 1988:243–268.

33. Esterbauer, H. S.; Striegl, G.; Puhl, H.; Oberreither, S.; Rotheneder, M.; El-Saadani, M.; Jurgens, G. The role of vitamin E and carotenoids in preventing oxidation of low-density lipoprotein. *Ann. NY. Acad Sci.* 570:254–67; 1989.
34. Regnstrom, J. N.; Nilsson, J.; Tornavall, P.; Landou, C.; Hamsten, A. Susceptibility to low-density lipoprotein oxidation and coronary atherosclerosis in man. *Lancet* 339:1183–1186; 1992.
35. Cominacini, L.; Garbin, U.; Pastorino, M.; Davoli, A.; Campagnola, M.; De Santis, A.; Pasini, C.; Faccini, G. B.; Trevisan, M. T.; Bertozzo, L.; Pasini, F.; Lo Cascio, V. Predisposition to LDL oxidation in patients with and without angiographically established coronary artery disease. *Atherosclerosis* 99:63–70; 1993.
36. Maggi, E.; Marchesi, E.; Ravetta, V.; Falaschi, F.; Finardi, G.; Bellomo, G. Low-density lipoprotein oxidation in essential hypertension. *J. Hypertension* 11:1103–1111; 1993.
37. Marui, N.; Offermann, M. K.; Swerlick, R.; Kunsch, C.; Rosen, C. A.; Ahmad, M.; Alexander, R. W.; Medford, R. M. Vascular cell adhesion molecule-1 (VCAM-1) gene transcription and expression are regulated through and antioxidant-sensitive mechanism in human vascular endothelial cells. *J. Clin. Invest.* 92:1866–1874; 1993.
38. Weber, C.; Erl, W.; Pietsch, A.; Strobel, M.; Loms Ziegler-Heitbrock, H. W.; Weber, P. C. Antioxidants inhibit monocyte adhesion by suppressing nuclear factor-κB mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulate to generate radicals. *Arterioscler Thromb.* 14:1665–1673; 1994.

ABBREVIATIONS

CD—conjugated dienes
EDTA—ethylenediamine tetraacetic acid
FCS—fetal calf serum
HUVECs—human umbilical vein endothelial cells
ICAM-1—intercellular cell adhesion molecule-1
LDL—low density lipoprotein
NF-κB—nuclear factor-κB
ODFR—oxygen-derived free radicals
PBS—phosphate-buffered saline
PLA_2—phospholipase A_2
ROIs—reactive oxygen intermediates
SLO—soybean lipoxygenase
TBA—thiobarbituric acid
TBARS—thiobarbituric acid reactive substances
TNF—tumor necrosis factor α
VCAM-1—vascular cell adhesion molecule-1